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THE RESTRICTION ENDONUCLEASE CLEAVAGE MAP OF RAT LIVER MITOCHONDRIAL DNA

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Summary

Mitochondrial DNA from rat liver contains six sites for cleavage by the restriction endonucleases Hind III and EcoRI. A large stretch of DNA, comprising about 40% of the mitochondrial genome is not cleaved by either of the enzymes; eight cleavage sites are located on a DNA stretch of 35% of the genome length suggestive of an unequal distribution of the A · T basepairs over the molecule. The number of Hind III and Eco R I fragments is much higher than reported for other mammalian mitochondrial DNAs up to now.

Introduction

Many of the initial observations with respect to mitochondrial nucleic acid and protein synthesis have been made using rat liver mitochondria as the experimental system [1]. With respect to mitochondrial DNA structure, replication and transcription, attention has recently been paid primarily to cultured mammalian cells and information about the number of cleavage sites for the restriction endonucleases stems mainly from studies with cells in tissue culture. In these studies it has been found that mtDNA from monkey-, mouse- and human cells is cleaved into one large fragment and one or two small fragments by the endonucleases Eco R I and Hind III [2,3]. This pattern of fragmentation by a single enzyme is not quite suitable for physical mapping of mitochondrial gene products by hybridization procedures since the resolution is limited while one fragment contains 80—90% of the genome length, so that double digestion should be indicated. Because of our interest in studying the biogenesis of mitochondria in laboratory animals, we have treated rat liver mitochondrial DNA with the restriction endonucleases Eco R I and Hind III. It is reported here that rat liver mitochondrial DNA contains six cleavage sites for either of the enzymes. The physical map of these fragments is presented.

Methods

Preparation of mitochondrial DNA from rat liver

Male albino rats (Wistar strain) weighing about 120 g were used after fasting overnight. Six livers were pooled, cut into small peices with scissors and homogenized with a glass-teflon Potter Elvehjem homogenizer using 0.25 M sucrose as the medium. Mitochondria were prepared by differential centrifugation as described previously [4]. The final mitochondrial pellet was resuspended to a volume of 12 ml in a medium containing 50 mM Tris buffer, 10 mM NaCl and 10 mM EDTA, pH 7.5. The further procedure for treating the mitochondria and isolating the closed circular fraction of the mtDNA was exactly as described by Bogenhagen and Clayton [5], except that the lower band of the CsCl/ethidium bromide density gradient was collected by suction into a plastic syringe after puncturing the centrifuge tube 1—2 mm below the band. These operations were performed under ultraviolet light to visualize clearly the DNA bands by their fluorescence emission. The DNA from 3 or 6 bands was combined, the ethidium bromide was removed by three successive extractions with an equal volume of isoamyl alcohol, the DNA solution was then diluted twice with 10 mM Tris/0.1 mM EDTA, pH 7.5 and the DNA precipitated in the cold with 2 vols. ethanol and 0.1 vol. of 1 M sodium acetate, pH 5.0. The DNA precipitate was collected by centrifugation (30 min, 15 000 × *g*) and dissolved in a small volume of 10 mM Tris/0.1 mM EDTA. The yield from six livers was about 250 μg mtDNA with an 230 : 260 : 280 absorbance ratio close to 0.5 : 1.0 : 0.5.

Enzymatic fragmentation and gel electrophoresis of mitochondrial DNA from rat liver

The rat liver mitochondrial DNA was digested with the restriction endonucleases Eco R I and/or Hind III at 37° C for different times in a medium containing 10 mM Tris · HCl, 10 mM $MgCl_2$, 150 mM NaCl and 5 mM mercaptoethanol; the final pH was 7.6. Eco R I and Hind III were obtained from Miles Laboratories Ltd. The Eco R I was diluted 1 : 50 from a stock solution with 10 mM sodium phosphate buffer, pH 7.0, containing 7 mM mercaptoethanol, 1 mM EDTA, 200 mM NaCl and 0.2% (v/v) NP_{40} (=Nonidet). The reactions were stopped by adding 0.3 vol. of a 70% sucrose solution in 100 mM EDTA, pH 7.0.

Gel electrophoresis was performed using slabgels of 20 × 30 cm, consisting of a small sealing layer of 10% acrylamide, a layer of 8—10 cm of 3% acrylamide and a layer of about 20 cm of 0.7% agarose. The gels were dissolved in a medium containing 40 mM Tris/acetate buffer, pH 7.8, 20 mM sodium acetate and 2 mM EDTA. Electrophoresis was for 15 h at room temperature and 120 V in the same buffer. The further details, the handling and photography of the slabgels were as described by Sanders et al. [6].

Results

Fig. 1 shows the fragment patterns of mtDNA from rat liver after fairly complete (Fig. 1A and C) and partial (Fig. 1B and D) digestion with the restriction

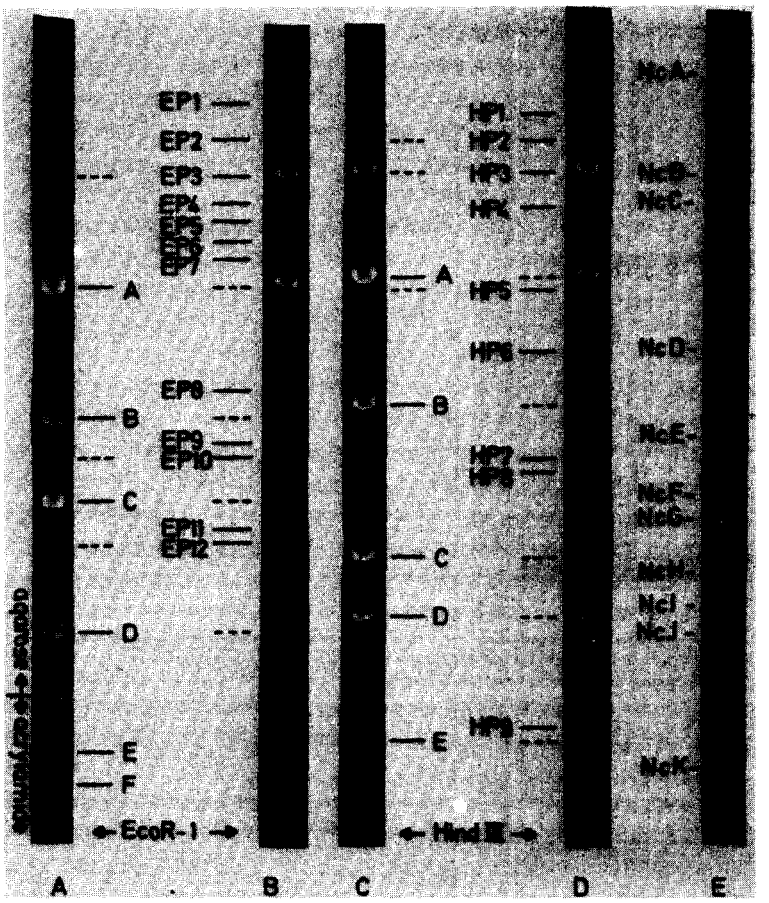

Fig. 1. Slabgel electrophoresis of fragments of mitochondrial DNA from rat liver. Samples of 3—5 μg mitochondrial DNA were treated with varying amounts of restriction endonuclease at 37°C for different times and electrophoresed on agaroseacrylamide slabgels. The details are given in the Methods section. Representative patterns from one slabgel with 16 slots are shown. The positions of the bands in the gels are further indicated by the bars in the columns next to the photographs. The interrupted lines show the positions of partials in A and C and of endfragments in B and D. (A) "complete" digest of Eco R I; (B) partial digest of Eco R I; (C) "complete" digest of Hind III; (D) partial digest of Hind III; (E) Complete Eco R I digest of *N. crassa* mitochondrial DNA. The molecular weights of the fragments NcA to NcK are 19 000, 10 500, 9000, 4500, 3450, 2850, 2700, 2250, 2000, 1875 and 425, respectively. EP = partial fragment from Eco R I digest; HP = partial fragment from Hind III digest.

enzymes Eco R I (Fig. 1A and B) and Hind III (Fig. 1C and D). The patterns shown were obtained in the same experiment in which, unfortunately, digestion did not go to completion. In Fig. 2 three further gels and densitometer tracings are shown to illustrate that in the complete digests six end-fragments can be detected in the Eco R I digest and five in the Hind III digest. No double peaks are present in either case.

The Eco R I fragments of *Neurospora crassa* mtDNA [7] and of ϕ 29 DNA [8] were used for calibration. The molecular weights of the different mtDNA fragments are given in Table I. The length of the Eco R I fragments A to E agrees well with the electron microscopical length measurements of Koike et al.

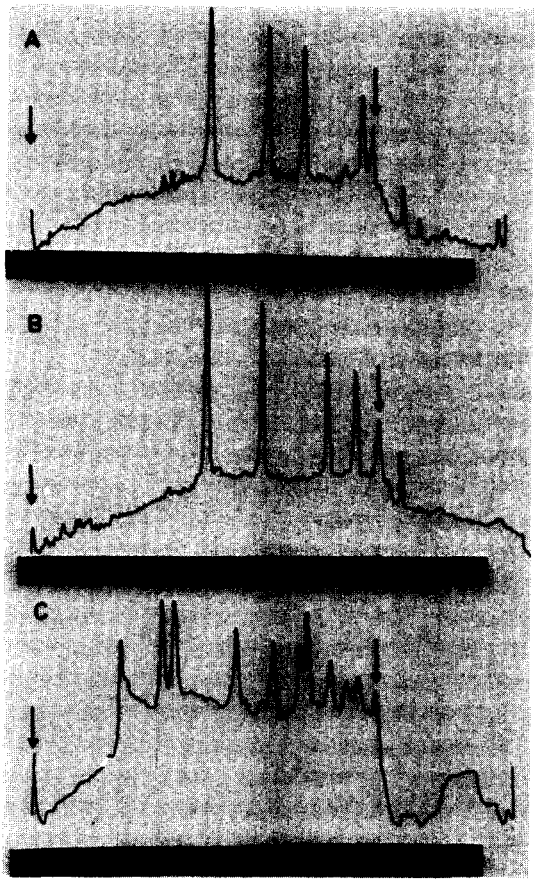

Fig. 2. Slabgel electrophoretograms and densitometer tracings of fragments of mitochondrial DNA from rat liver. (A) Eco R I digest and tracing of rat liver mtDNA; (B) Hind III digest and tracing of rat liver mtDNA; (C) Eco R I digest and tracing of *Neurospora* mtDNA. The arows at the left indicate the position of the top of the gel; the arrows at the right indicate the position of the boundary between the agarose and polyacrylamide gel layers. For further details see the legend to Fig. 1.

TABLE I

LENGTH OF THE RESTRICTION ENZYME FRAGMENTS OF RAT-LIVER MITOCHONDRIAL DNA

For experimental details see the Methods section.

Fragment	Length of fragment (nucleotide pairs)	
	Eco R I	Hind III
A	5500	5950
B	3550	3750
C	2650	2300
D	1800	1900
E	650	800
F	400	150
Σ	14550	14850

[9]. These authors did not report data on the length of a fragment comparable to our Eco R I fragment F. The total length of the fragment is about 14 500 basepairs. This is in good agreement with the 10×10^6 molecular weight of mitochondrial DNA from rat liver [10]. It further excludes gross heterogeneity of the mitochondrial DNA. From Fig. 1B, it can be concluded from the double band between fragments C and D that the two small fragments present in the Eco R I digest must be adjacent to fragment D. Similarly it can be concluded at first glance that the second fragment present in the acrylamide-gel layer in Fig. 1D must be a partial fragment that gives rise to the formation of fragment E on further digestion. The other fragment, F, is too small to be detected by the method used.

Of course we have considered the possibility that our enzymes were impure and that the higher number of fragments had to be attributed to contaminating endonucleolytic activity. This possibility was excluded by our observations that digestion of ϕ 29 DNA and *N. crassa* mitochondrial DNA with Eco R I gave the expected number of fragments. Moreover, different batches of the enzymes gave exactly the same cleavage patterns. Finally we also prepared mouse liver mitochondrial DNA. We found the same cleavage pattern for this DNA as that previously reported for mouse L cells [2,3] (Fig. 3). Eco R I (Fig. 3B) gives one large and one small fragment, Hind III (Fig. 3C) one large and two small fragments. In the cross digest (Fig. 3A) five fragments could be detected.

The order of the fragments on the rat mitochondrial genome has been esti-

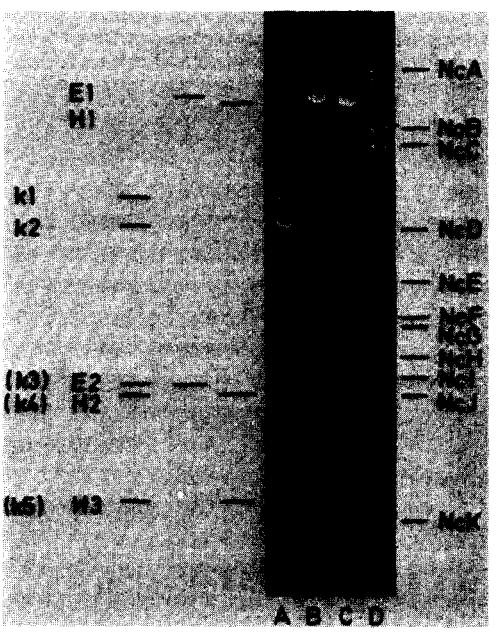

Fig. 3. Slabgel electrophoresis of fragments of mitochondrial DNA from mouse liver. Mouse liver mitochondrial DNA was prepared from the livers of 15 albino mice by exactly the same method as described for six rat livers in the Methods section. Samples of about 3 μg DNA were analyzed on slabgels. (A) cross digest of Eco R I plus Hind III (K1—K5); (B) complete digest of Eco R I (E1—E2); (C) complete digest of Hind III (H1—H3); (D) Complete Eco R I digest of *N. crassa* mitochondrial DNA (NcA—NcK).

TABLE II

LENGTH AND INFERRED COMPOSITION OF THE PARTIAL DIGESTION PRODUCTS OF RAT LIVER MITOCHONDRIAL DNA, OBTAINED WITH THE RESTRICTION ENDONUCLEASES ECO R I AND HIND III

For experimental details see the Methods section. The fragment annotation is the same as in Fig. 1. The fragments EP 1, EP 2, HP 1 and HP 2 represent full-length molecules in the linear and open circular form and are omitted from the table. The vague band EP 6 could not be identified in this way.

Eco R 1			Hind III		
Fragment	Length (basepairs)	Composition	Fragment	Length (basepairs)	Composition
EP 3	10600	A + C + D + E	HP 3	10700	A + C + D + E + F
EP 4	9100	A + B	HP 4	8700	A + C + E
EP 5	8200	A + C	HP 5	5650	B + D
EP 6	7100	—	HP 6	4300	B + E + F
EP 7	6400	B + D + E + F	HP 7	3200	C + E + F
EP 8	3900	B + F	HP 8	3100	C + E
EP 9	3300	C + E	HP 9	900	E + F
EP 10	3100	D + E + F			
EP 11	2550	D + E			
EP 12	2450	D + F			

mated on the basis of the molecular weights of the partial fragments in the incomplete digests. Due to, on the one hand the fact that our calibrations in the low molecular weight range are not very accurate, and at the other hand the

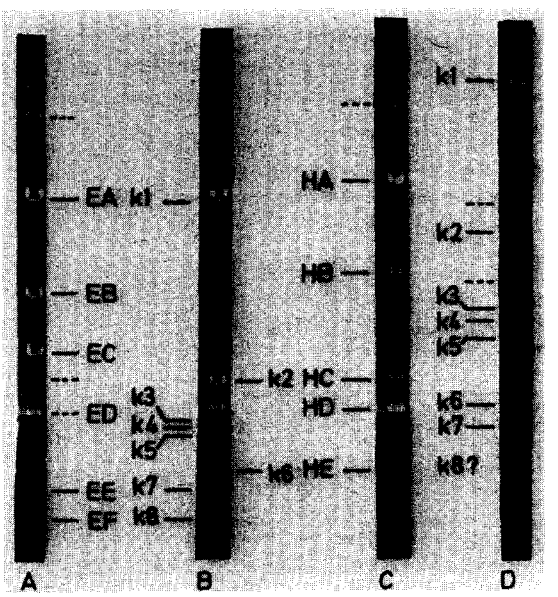

Fig. 4. Slabgel electrophoresis of fragments of mitochondrial DNA from rat liver. All details and annotations are as in Fig. 1. (A) Eco R I digest; (B) combined Eco R I plus Hind III digest; (C) Hind III digest; (D) as B but from another preparation and another slabgel with all fragments present in the 0.7% agarose-gellayer.

difference of only about 400 basepairs between the Hind III fractions C and D, the order of the Hind III fragments could not be estimated unequivocally on this basis. Two possible arrangements were left: ADBFECA and ACBFEDA. For the Eco R I fragments the order of the fragments is ACEDFBA. Table II gives a summary of the data from different experiments that have led to the construction of the physical map of the fragments. Further, we looked for the fragment pattern of a double digest using both enzymes together. The results are shown in Fig. 4. Eight fragments could be detected. It can be concluded that the Hind III A and Eco R I A fragments are grossly overlapping. Fragments similar to the Hind III fragments C and E and the Eco R I fragments E and F were present in the cross digest. In a separate experiment we prepared the Eco R I fragments A—D and subsequently cleaved these isolated fragments with Hind III. The following results were obtained: Eco R I A was shortened slightly; Eco R I B gave rise to a fragment of the Hind III C-size and some smaller fragments since two partials could be detected; Eco R I C was cleaved into two almost equal parts. Also, Eco R I fragment D was cleaved, although only one fragment of about 1400 basepairs was recovered.

Discussion

From the data obtained in the different experiments shown above, we have constructed the physical map as illustrated in Fig. 5. The 12 restriction sites of the two enzymes used are divided unequally over the genome. 35% of the genome length is not cleaved at all, whereas eight cleavage points are located on a DNA stretch of 32% of the total DNA. Since both enzymes are specific for hexanucleotide pairs containing 4 A · T basepairs, one might assume that the A · T basepairs are not randomly distributed over the DNA molecule. This is in agreement with the electron microscopical observations with partially heat-denatured mitochondrial DNA from rat liver [11] and *Drosophila melanogaster* [12]. Also for lower eukaryotes unequal distribution of AT-basepairs has been reported [6,13,14].

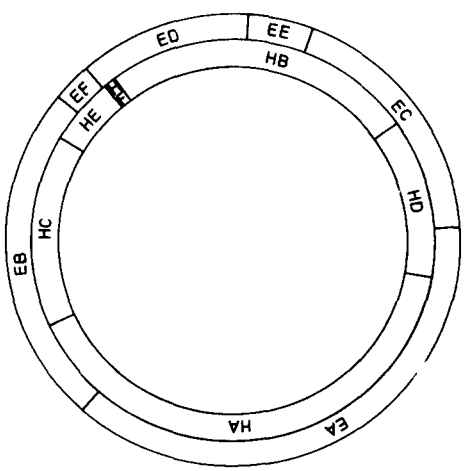

Fig. 5. The physical map of rat liver mitochondrial DNA.

The manner in which rat liver mtDNA is cleaved into six fragments by either of the enzymes renders it more suitable than the animal mtDNAs described so far for further studies on the localization of mitochondrial transcription products and other genetic markers such as the origin of replication and the recognition sites for the various polymerases. However, it must be recognized that the small fragments are very difficult to prepare on a sufficient scale and may even be missed completely if inadequate methods for separation of the fragments are used. This is especially inconvenient in case small transcription products such as the mitochondrial tRNAs are to be localized on the mitochondrial genome.

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